

## Heterostyly in *Linum aretioides*

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**Abstract:** The petal width; sepal, pistil, and stamen lengths; and changes in pollen sizes associated with heterostyly of different morphs in populations of *Linum aretioides* Boiss. were investigated. Fruit sets were compared in 2 populations and it was investigated whether the Bozdağ population has a heteromorphic incompatibility system. It was observed that the stamen lengths of thrum morphs of Bozdağ were shorter than those of thrum morphs of Babadağ. Pistil lengths of both morphs of Bozdağ were longer than those of morphs of Babadağ. Pollen shapes of both morphs were prolate-spheroidal and subprolate, respectively, in the Bozdağ population and subprolate in the Babadağ population. It was determined that pollen exine in the short-styled and long-styled flowers was dimorphic. The pollen bore a marginal ring of 4 to 8 spinules in the thrum morphs and of 3 to 7 spinules in the pin morphs. It was found that the highest rates of fruit set occurred during cross-hand pollination from pin morphs to thrum morphs and from thrum morphs to pin morphs (77.77% and 71.42%, respectively). It was shown that in *L. aretioides* self-pollinations and pollinations between plants of the same morph are incompatible.

**Key words:** *Linum*, breeding systems, heterostyly, heteromorphic incompatibility

### 1. Introduction

A common associated feature of heterostyly is the presence of a self- and intramorph-incompatibility system that allows only legitimate (between anthers and stigmas of the same level) pollination to set fruit. For a species to be considered heterostylous, the floral morphs must differ reciprocally in stamen and style length (Lloyd & Webb, 1992). The reciprocal arrangement of male and female sexual organs may reduce pollen wastage by increasing legitimate pollination. Individuals in the majority of distylous populations are both self-sterile and intramorph sterile (Darwin, 1877; Kohn & Barrett, 1992; Wolfe, 2001). In distylous plants, the supergene determining floral morphology also controls a diallelic sporophytic self-incompatibility system, so that only pollinations between morphs are compatible (Nettancourt, 1977; Ganders, 1979; Beliveau & Wyatt, 1999; Barrett, 1992, 2002).

Differences in floral morphology are usually associated with an incompatibility system in which each morph setting seed in a plant is only pollinated by the other morph (Dulberger, 1992). The flowers of distylous species are often tubular (Beliveau & Wyatt, 1999).

Distyly has arisen independently in at least 28 angiosperm families (Barrett et al., 2000). One of these

families is Linaceae. Of the 49 taxa belonging to Linaceae in Turkey, 21 are endemic (Davis, 1967). Some of the *Linum* L. species in this family are heterostylous. Several studies have been carried out on the breeding system of *Linum* species (Ockendon, 1968; Dulberger, 1973, 1981; Rogers, 1979). Darwin (1877), one of the first researchers of this species, revealed the existence of distyly in several species such as *L. pubescens* Banks & Sol., *L. grandiflorum* Desf., *L. mucronatum* Bertol., *L. flavum* L., *L. perenne* L., *L. austriacum* L., and *L. maritimum* L.

Many studies have been conducted on the reproductive biology of rare plant species. Some of these studies are as follows. Subaşı and Güvensesen (2011) investigated the reproductive ecology, reproductive system, and reproductive success of the *Salvia smyrnaea* Boiss. population on Mount Nif. Melia et al. (2012) determined the optimum conditions for seed germination, development of seedlings, and ex-situ conservation and self-regeneration capacity of *Arbutus andrachne* L. and *Osmanthus decorus* (Boiss. & Balansa) Kasapligil. Oziegbe et al. (2012) defined the floral morphology and reproductive biology of *Ludwigia leptocarpa* (Nutt.) H.Hara and *L. adscendens* (L.) H.Hara, in order to determine the major characteristics necessary for their spread.

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The current study aimed to investigate polymorphism in pistil and stamen lengths of different morphs and fruit set in 2 populations. Another aim was to investigate changes in pollen sizes associated with heterostyly in the populations of *Linum aretioides* Boiss., a distylic species placed in the vulnerable (VU) category according to the *Red Data Book of Turkish Plants* prepared by Ekim et al. (2000) but which was considered to be in the critical (CR) category in a later study performed by Seçmen et al. (2006). Our study also aimed to examine whether the Bozdağ population of *L. aretioides* showed a heteromorphic incompatibility system.

## 2. Materials and methods

### 2.1. The study site

*Linum aretioides*, a local endemic, is distributed only on 2 mountains (İzmir: Bozdağ and Denizli: Babadağ), accruing in the western part of Turkey between the altitudes of 1630 and 2300 m, above the tree zone. The studies were conducted on Bozdağ between 1750 m and 2150 m, and on Babadağ between 1630 m and 2300 m (Figure 1). Average annual temperatures vary between 7 °C and 9 °C at 2200 m on Bozdağ and between 5.7 °C and 6.0 °C at 2300 m on Babadağ. Average annual rainfall on Bozdağ's summit was calculated as 1125.9 mm and on Babadağ's summit 1139.6 mm using extrapolation charts according to the 31-year average precipitation values. Soils are classified as high mountain meadow soils and alpine mountain meadow soils, and are usually lithosolic (containing high amounts of gravels). Over the regions where the plant is distributed, soils have the characteristics of Palaeozoic schist fine-grained gneiss on Bozdağ and the characteristics of anthracolitic Carboniferous-Permian limestone (anthracolitic schist and sandstone, and crystalline limestone) on Babadağ (Atalay, 1982).

### 2.2. Study species

*Linum aretioides* is a perennial semi-shrub, and mostly consists of round or roundish tufts. It has thick roots and multiple stems. Its leaves are 0.55–1.30 × 0.2 cm, linear or linear-setaceous, and they have a midrib. A large number of sterile and fertile short shoots stay together. A single sessile flower blooms at the tip of the fertile shoots (Figure 2). The flowers are distylic and yellow (Figure 2). Flowering occurs during May and June, whereas seed set takes place during July and August.

There are 5 stamens, the bottom parts of the filaments are attached to each other, and the anthers are attached to filaments basifixed. Nectar is secreted between the inner surface of the petal and the outer surface of the filament at the bottom of the filaments. There are nectar guides on petals that help insects to reach the nectar present in the nectar pits. The ovary has 5 carpels, which adds up to 10 with false compartments. Styli in flowers have 5 pieces. Stigmas, which are linear, papillate, and wet both in thrum morphs and in pin morphs, are longer and wider in pin morphs but smaller and narrower in thrum morphs. Natural populations of *L. aretioides* consist of 2 floral morphs: long styled (pin) plants with short stamens and short styled (thrum) plants with long stamens.

Individuals are either thrum morphs or pin morphs. The 5 petals are 4.0–1.4 × 0.22 cm. Petals consist of a broad obovate lamina and a claw entering the calyx. Flowers shed their petals in a very short time. The 5 ovate sepals with pointed tips and membranous edges are 0.5–0.6 cm. Fruits are orbiculate-ellipsoid and 0.53–0.69 × 0.26–0.50 cm, and they have obtuse or beak-like projections. The seeds are 0.30–0.48 × 0.11–0.19 cm, oval, and compressed laterally.

### 2.3. Floral morphometry

In order to conduct morphometric analysis, samples were

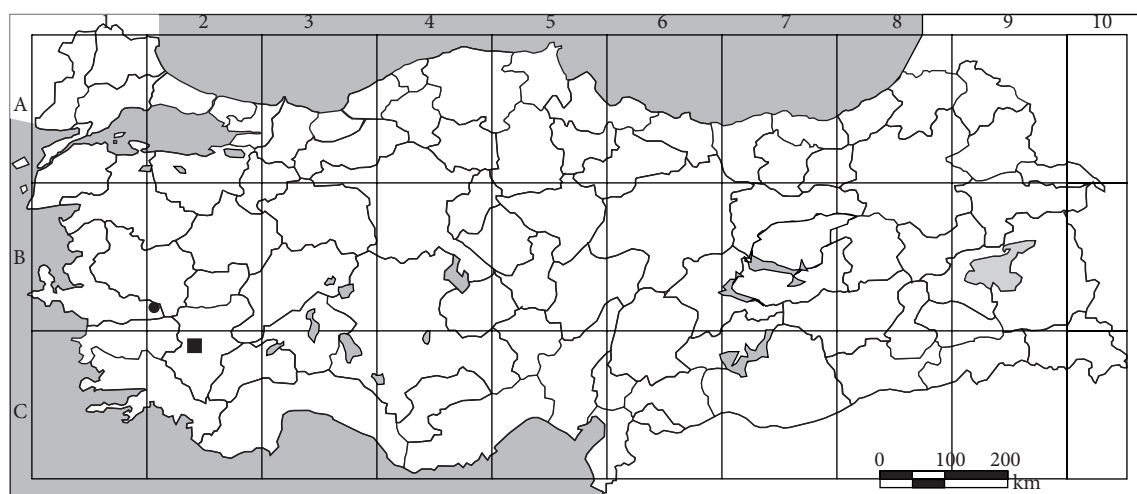
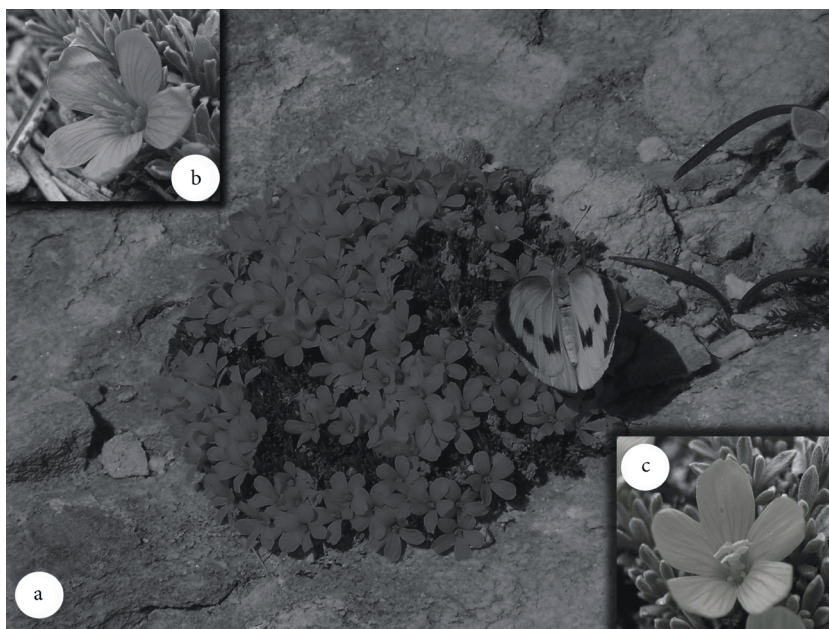


Figure 1. Map showing the study area [Bozdağ (●) and Babadağ (■)] and its location in Turkey.



**Figure 2.** *Linum arctioides*: (a) habit; (b) pin morph; (c) thrum morph.

collected on 7–8 June 2006 from Bozdağ and on 30–31 May 2006 from Babadağ. Flower samples of different morphs were collected randomly from the morphs of both populations. One sample was taken from each plant. On Bozdağ and Babadağ, 50 samples were collected for petal width and petal length measurements. On Bozdağ, 20 flowers from pin morph individuals and 30 flowers from thrum morph individuals were collected for stamen height and pistil height measurements. On the other hand, on Babadağ, 30 flowers from thrum and pin morph individuals were collected for stamen height and pistil height measurements. The flowers collected were preserved in 70% ethanol, transported to the laboratory, and measured with a handheld digital scanning electron microscope (SEM) (in millimetres).

Pollen characters of each population were determined in 100 grains of 1 stamen from 10 randomly selected individuals of each morph. Slides were prepared in accordance with Wodehouse's (1965) method. Pollen grains were measured under a PRIOR binocular light microscope using a micrometric ocular with  $\times 10$  and  $\times 40$  objectives. Polar axis (P), equatorial axis (E), colpus width (clt), colpus length (clg), one side of the polar triangle, and intine, exine, endexine, and ektexine thickness of the pollen were measured. In addition, a JEOL JSN 6060 scanning electron microscope was used to observe exine ornamentation of the pollen to obtain micrographs. The pollen terminology described by Hesse et al. (2009) was used.

#### 2.4. Bagging and crossing experiments

The mating system of the species and the importance of insects as pollinating agents were investigated by bagging

and crossing experiments on 7–8 June 2006 on Bozdağ. The following experiments were carried out:

The letter "N" represents the number of flowers worked on. At the end of these experiments, the percentages of fruit set were recorded.

a) Self-pollination was achieved in thrum morphs (N = 19) and pin morphs (N = 30) with a brush and the flowers were bagged with fine nylon mesh (autogamy).

b) Pollen transfer from the stamens of thrum morphs (N = 14) to the stigmas of pin morphs (N = 14) and from the stigmas of pin morphs (N = 18) to the stigmas of thrum morphs (N = 18) was carried out with a brush and then the flowers were bagged with fine nylon mesh (allogamy).

c) Thrum morphs (N = 9) and pin morphs (N = 11) were bagged with fine nylon mesh, when they were still in bud (cleistogamy).

d) When thrum morphs (N = 10) and pin morphs (N = 10) were still in bud, their anthers were cut and the morphs were bagged with fine nylon mesh (apomixis).

e) Thrum morphs (N = 25) and pin morphs (N = 25) were bagged with fine nylon mesh (entomogamy).

f) Thrum morphs (N = 432 in 26 individuals) and pin morphs (N = 152 in 16 individuals) morphs were left unbagged throughout the flowering period (natural control).

Approximately 1 month later, percentages of fruit set were determined in all the experiments.

#### 2.5. Statistical analysis

The differences between morphs and populations were tested by one-way ANOVA. Significant differences among means were determined by Student's t test. The relationship

between pistil and stamen heights was analysed by a simple correlation test. All tests were performed at the significance level of  $\alpha = 0.05$  with SPSS v. 11.5 (SPSS Inc., Chicago, IL, USA).

### 3. Results

#### 3.1. Floral morphology

The flowers were dimorphic, being either pin morphs or thrum morphs (Figure 3). Mean  $\pm$  SD values of 4 floral

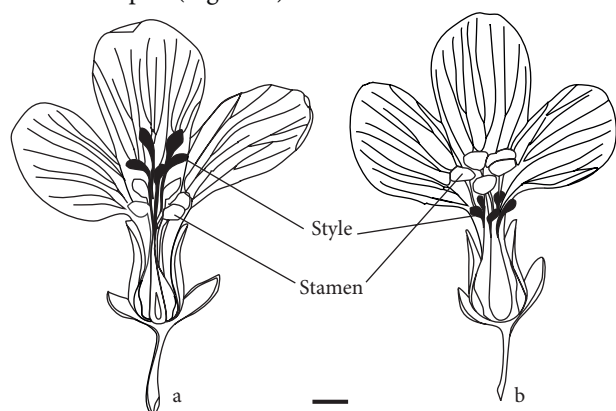


Figure 3. (a) Pin morph; (b) thrum morph. Scale bar = 1 mm.

characters and the mean  $\pm$  SD are shown in Table 1. Measurements of petal widths and lengths of thrum and pin morphs of Bozdağ and Babadağ plants revealed that petal widths and lengths of thrum and pin morphs of Bozdağ were significantly larger than those of Babadağ. It was also determined that the differences between petal widths and lengths of thrum and pin morphs of Bozdağ and those of Babadağ and the differences between petal lengths of thrum and pin morphs of Babadağ were significant ( $P < 0.001$ ) (Table 1).

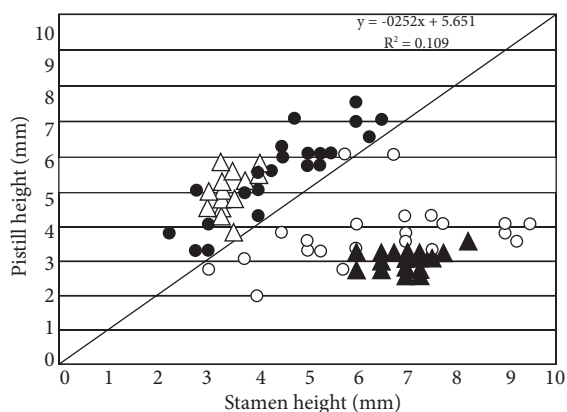
When the stamen and pistil lengths of similar morphs of both populations were compared, it was observed that the stamen lengths of thrum morphs of Bozdağ were shorter than those of Babadağ, whereas the stamen lengths of pin morphs of Bozdağ were longer than those of Babadağ. Pistils of both morphs of Bozdağ were longer than those of Babadağ. Statistically significant differences were determined between the stamen and pistil lengths of the flowers of both populations (Table 1; Figure 4).

Artificial pollinations were carried out to determine whether an incompatibility system is associated with the floral dimorphism. Fruit production was recorded following self- and intramorph cross-pollination

**Table 1.** Comparison of 4 floral characters between flowers from thrum morph and pin morph plants of *Linum arretiooides*.

Floral characters	Thrum morph	Pin morph	<i>t</i> (short:long)
Petal width (mm)			
Bozdağ	4.05 $\pm$ 0.16	3.86 $\pm$ 0.18	0.19 (NS)
Babadağ	3.17 $\pm$ 0.09	3.23 $\pm$ 0.12	0.06 (NS)
<i>t</i> (Bozdağ:Babadağ)	0.88***	0.63***	
Petal length (mm)			
Bozdağ	10.83 $\pm$ 0.24	9.59 $\pm$ 0.47	1.24 (NS)
Babadağ	9.63 $\pm$ 0.16	7.97 $\pm$ 0.47	1.66***
<i>t</i> (Bozdağ:Babadağ)	1.20***	1.62***	
Stamen height (mm)			
Bozdağ	6.38 $\pm$ 0.31	4.65 $\pm$ 0.2	1.73***
Babadağ	6.85 $\pm$ 0.08	3.27 $\pm$ 0.07	3.58***
<i>t</i> (Bozdağ:Babadağ)	0.47 (NS)	1.38***	
Pistil height (mm)			
Bozdağ	3.51 $\pm$ 0.16	6.03 $\pm$ 0.19	2.52***
Babadağ	3.07 $\pm$ 0.04	5.03 $\pm$ 0.09	1.96***
<i>t</i> (Bozdağ:Babadağ)	0.44**	1.00***	

(Data are given as the mean  $\pm$  SD. Sample sizes were as follows: for petal width and petal length 50 flowers from thrum morph individuals and 50 flowers from pin morph individuals on Bozdağ and Babadağ; for stamen height and pistil height 20 flowers from pin morph individuals and 30 flowers from thrum morph individuals on Bozdağ, and 30 flowers from thrum and pin morph individuals on Babadağ. *t*, value of Student's *t*-test. NS, not significant. \*\*  $P < 0.01$ , \*\*\*  $P < 0.001$ ).



**Figure 4.** Correlation between pistil and stamen heights of *L. aretioides* (thrum morph (○) and pin morph (●) specimens from Bozdağ; thrum morph (▲) and pin morph (△) specimens from Babadağ).

(illegitimate pollinations), and the results were compared with those from intermorph pollinations (legitimate pollinations). The results for the different pollinations are summarised in Table 2.

Fruit set did not occur in (c), (d), or (e) test experiments. However, the highest rates of fruit set occurred in test (b) during cross-hand pollination from pin morphs to thrum morphs and from thrum morphs to pin morphs (77.77% and 71.42%, respectively). It was also found that the results obtained from (f) experiments in the control group were close to the results obtained from (b) experiments in the cross-hand pollination group. The rates of fruit set in (a) experiments in the self-hand pollination group were very low (Table 2).

### 3.2. Pollen features

When the morphological features of the pollen of thrum and pin morphs in both populations were evaluated, it was determined that pollen grains were tricolpate, colpi had straight edges and pointed tips, and operculum was located in the middle of a colpus and was granulated. Pollen structure was tectate, whereas pollen sculpture was baculate. Upper surface widths of baculum were different, had 4–8 spinules, and the distances between spinules were irregular. In the Bozdağ population, in thrum morphs and pin morphs, P/E was 1.08 and 1.16, respectively. Pollen of thrum morphs and pin morphs was prolate-spheroidal and subprolate, respectively. In the Babadağ population, P/E was 1.27 in thrum morphs and 1.28 in pin morphs, and the pollen of both morphs was subprolate. Polar ends appear obtuse in thrum morph pollen and round in pin morph pollen (Figure 5).

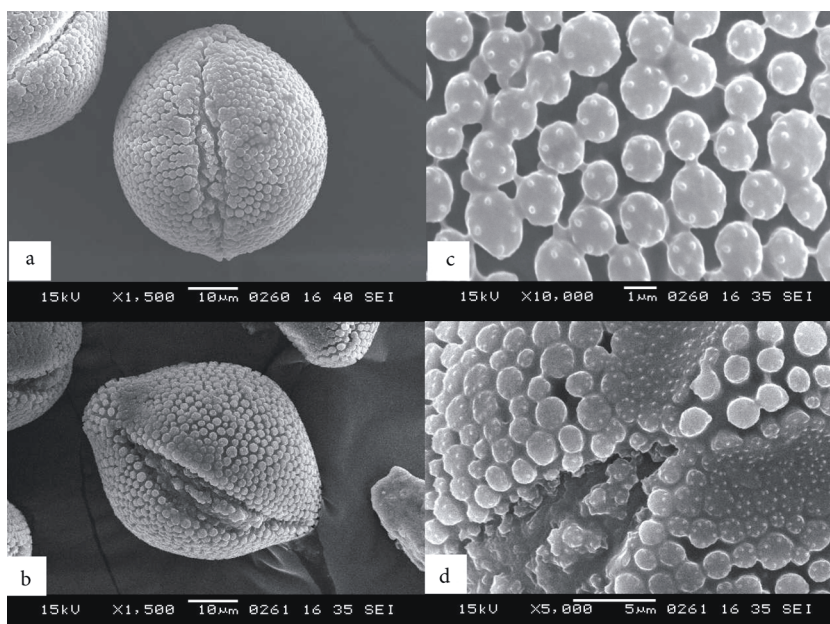
When the exine characteristics of pollen of thrum morphs and pin morphs of *L. aretioides* were evaluated under the SEM, it was determined that the pollen of thrum morphs bore a marginal ring of 4 to 8 papillae, but the pollen of pin morphs bore a marginal ring of 3 to 7 papillae (Figure 5).

### 4. Discussion

For successful pollination in heterostylous plants, there should be pollen and stigma compatibility between the morphs and pollen and stigmas should be positioned reciprocally. Differences may lead to complications. For pollination proficiency, the persistence of distyly depends on tight linkage of the genes affecting anther and stigma heights (Kohn & Barrett, 1992; Lewis & Jones, 1992). The data we obtained revealed significant differences between

**Table 2.** Fruit set from the 5 experiments and controls in *L. aretioides* [S: thrum (short styled flowers), L: pin (long styled flowers), N: number of flowers examined].

Treatments	Style	N	Fruit set (%)
(a) Self hand pollination	S → S	19	3 (15.78)
	L → L	30	4 (13.33)
(b) Cross hand pollination	S → L	14	10 (71.42)
	L → S	18	14 (77.77)
(c) Cleistogamy	S	9	0
	L	11	0
(d) Apomixis	S	10	0
	L	10	0
(e) Entomogamy	S	25	0
	L	25	0
(f) Open pollinated (controls)	S	432	307 (71.06)
	L	152	111 (73.02)



**Figure 5.** Equatorial and surface view of pollen of different morphs in SEM. Thrum morph: (a: equatorial view, c: ornamentation); Pin morph: (b: equatorial view, d: ornamentation).

the different morphs of Bozdağ and Babadağ. In reciprocal reproductive organs of Bozdağ morphs, stamen (thrum morph)–pistil (pin morph) difference is 0.35 mm and stamen (pin morph)–pistil (thrum morph) difference is 1.14 mm. These differences are 1.82 mm and 0.20 mm, respectively, in Babadağ morphs (Table 1; Figure 3). Based on these values calculated, it can be stated that stamen (pin morph)–pistil (thrum morph) difference in Bozdağ morphs ( $P < 0.001$ ) and stamen (thrum morph)–pistil (pin morph) difference in Babadağ morphs ( $P < 0.000$ ) are statistically significant.

Petal lengths in many heterostylous species (e.g., *Jepsonia* Small, *Oxalis* L., herbaceous Rubiaceae) were <10 mm long. In a few cases, there were differences in the size of the corolla between pin and thrum morphs. Rather few distylous species were dimorphic for corolla size, and, in all cases, thrum corollas were larger than pin corollas (Ganders, 1979). Thrum morphs were larger than pin morphs in some species of *Lithospermum* L., such as *L. densiflorum* Ledeb. ex Nordm. (Johnston, 1952) and *L. caroliniense* (J.F.Gmel.) MacMill. (Levin 1968), *Amsinckia vernicosa* Hook. & Arn. var. *furcata* (Suksd.) Hoover ex Jeps., *A. douglasiana* A.DC. (Ganders, 1976), *A. grandiflora* Kleeb ex Greene (Ornduff, 1976), *Fagopyrum esculentum* Moench (Darwin, 1877), and *Rudgea jasminoides* (Cham.) Müll.Arg. (Baker, 1955). In the present study petals of the thrum morphs were longer than those of pin morphs in both populations (Table 1).

Proportional imbalance between flowers and morphs accelerates the population decline. It was determined

that thrum morphs were more than pin morphs in both populations. While the thrum morph/pin morph ratio (TM/PM) was 1.15 in the Bozdağ population, it was 1.02 in the Babadağ population. Nicholls (1986) reported that in *Linum perenne* thrum morphs produce more pollen, pin morphs set more seeds, and thrum morphs serve the function of a male and pin morphs the function of a female.

There is often a suite of morphological dimorphisms (e.g., pollen size, pollen production, sculpture of pollen walls, and stigmatic papillae size) that accompany the style and stamen length (Dulberger, 1992). In several studies on *Linum* species, almost no difference was observed in the stamen lengths. In *Linum suffruticosum* L., dimorphism was reported in the style length and pollen exine ornamentation (Rogers, 1979). In *L. grandiflorum* pollen dimorphism was found (Saad, 1961; Erdtman, 1964; Dulberger, 1981). According to Dulberger (1974), dimorphic exine ornamentation was observed in *L. grandiflorum*, *L. austriacum*, *L. perenne*, *L. pubescens*, *L. mucronatum*, *L. flavum*, and *L. maritimum*. In our study, the pollen of short-styled morphs bears a marginal ring of 4 to 8 spinules and the pollen of long-styled morphs bears a marginal ring of 3 to 7 spinules in *L. aretioides*. It was determined that pollen exine in the short-styled and long-styled flowers was dimorphic.

The most common polymorphisms are differences in size between pins and thrums, while less common ones are differences in shape, colour, exine ornamentation, or in the presence or absence of starch in the pollen grain (Ganders,

1979). In many distylous species, thrums produce larger pollen grains (Dulberger, 1992; McKenna, 1992). Anther height is correlated positively with pollen size and negatively with papilla size, while stigma height is correlated negatively with pollen size and positively with papilla size (Dulberger, 1974; Li & Johnston, 2001). Ganders (1979) suggests that the reduced size of pollen grains of the LS flowers (pin morphs) could be an adaptation that enhances transfer probabilities toward the less accessible stigmas of SS flowers (thrum morphs). Ree (1997) suggests that the larger stigma in SS flowers (thrum morphs) is an adaptation that increases the probabilities of pollen receipt. In our findings, pollen sizes of thrums of *L. aretioides* are  $58.88 \pm 0.88$  in Bozdağ and  $59.60 \pm 0.77$  in Babadağ, which are bigger than pollen sizes of pin morphs. Dulberger (1992) reports that the ratio of thrum to pin pollen size in most distylous species varies from 1.06 to 1.80. In our findings, the ratio of thrum to pin pollen size (thrum/pin) in *L. aretioides* is 1.03 in Bozdağ and 1.09 in Babadağ. There are significant differences in the pollen sizes and exine ornamentation features of both morphs of *L. aretioides*.

Pollination experiments with *Linum mucronatum* and *L. pubescens* were conducted in a study by Dulberger (1973). In both species, fecundity was significantly higher in pin flowers than in thrum morphs. These results suggest that, in *L. mucronatum*, the fecundity of the 2 morphs differed as a result of illegitimate pollinations. Capsules developed in 25.5% of 43 pollinated pin flowers but in only 4% of 75 thrum morphs. In *L. pubescens*, illegitimate pollinations were traced in 45 thrum and 46 pin styles. A large number of fruits were found in thrum × thrum crosses and a smaller number in pin × pin crosses. In *L. pubescens*, illegitimate pollinations were ineffective, and thus only 4.2% of 141 illegitimately pollinated flowers produced capsules; in contrast, legitimate pollinations resulted in fruit production in 92.3% of 78 flowers. According to the results of pollination experiments performed in the field on *L. areti-*

*oides*, the highest fruit set rates were obtained as a result of intermorph pollination. In *L. aretioides* legitimate pollinations resulted in fruit production in 77.77% of 18 flowers (L→S) and 71.42% of 14 flowers (S→L). While fruit set rates obtained from open-pollination experiments were high as well, they were low in intramorph pollination experiments. In *L. aretioides*, fruit set after illegitimate pollinations did not differ between the 2 morphs. Capsules developed in 13.3% of 30 pollinated pin morphs and in 15.78% of 19 thrum morphs (Table 2). These experiments showed that, in *L. aretioides*, pins and thrums are compatible with each other, but self-pollinations and pollinations between plants of the same form are incompatible.

It has been reported that pollinators of bright yellow and red flowers are mostly butterflies (Glover, 2007). The flowers of *L. aretioides* are bright yellow and have floral nectariums. In a study conducted on this plant in both of the study fields, the most active species in pollination were butterflies, in particular *Pieris brassicae*. There are many pollinating insects in the areas where *L. aretioides* is distributed (Seçmen et al., 2010). In this case, there is no problem in terms of pollinators. The reason why pollinators cannot transfer enough pollen can be attributed to such factors as the pollinator species, or types and sizes of stigmas. Large stigmas of pin flowers can hold more pollen than smaller stigmas of thrum morphs. Butterflies have more convenient probosces than bees to obtain nectar from the holes between the overlapping petals in flowers, which may be the cause of the low fruit set. Bees hold larger amounts of pollen with their legs and probosces than do butterflies with their thin and long probosces. In this case, a small number of legitimate pollen grains in a butterfly's proboscis may contribute to the low fruit set.

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